AAC Journals.ASM.org

Effects of Tigecycline and Vancomycin Administration on Established *Clostridium difficile* Infection

Casey M. Theriot,[a] Cassie A. Schumacher,[b]* Christine M. Bassis,[a] Anna M. Seekatz,[a] Vincent B. Young[a,b]

Department of Internal Medicine/Division of Infectious Diseases[a] and Department of Microbiology and Immunology,[b] The University of Michigan, Ann Arbor, Michigan, USA

The glycylcycline antibiotic tigecycline was approved in 2005 for the treatment of complicated skin and soft tissue infections and complicated intra-abdominal infections. Tigecycline is broadly active against both Gram-negative and Gram-positive microorganisms, including *Clostridium difficile*. Tigecycline has a low MIC against *C. difficile in vitro* and thus may represent an alternate treatment for *C. difficile* infection (CDI). To assess the use of tigecycline for treatment of established CDI, 5- to 8-week-old C57BL/6 mice were colonized with *C. difficile* strain 630. After *C. difficile* colonization was established, mice ($n = 10$ per group) were treated with either a 5-day course of tigecycline (6.25 mg/kg every 12 h subcutaneously) or a 5-day course of vancomycin (0.4 mg/ml in drinking water) and compared to infected, untreated control mice. Mice were evaluated for clinical signs of CDI throughout treatment and at 1 week posttreatment to assess potential for disease development. Immediately following a treatment course, *C. difficile* was not detectable in the feces of vancomycin-treated mice but remained detectable in feces from tigecycline-treated and untreated control mice. Toxin activity and histopathological inflammation and edema were observed in the ceca and colons of untreated mice; tigecycline- and vancomycin-treated mice did not show such changes directly after treatment. One week after the conclusion of either antibiotic treatment, *C. difficile* load, toxin activity, and histopathology scores increased in the cecum and colon, indicating that *C. difficile*-associated disease occurred. *In vitro* growth studies confirmed that subinhibitory concentrations of tigecycline were able to suppress toxin activity and spore formation of *C. difficile*, whereas vancomycin did not. Taken together, these data show how tigecycline is able to alter *C. difficile* pathogenesis in a mouse model of CDI.

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus (1). *C. difficile* infection (CDI) causes a range of clinical disease, with more severe cases leading to pseudomembranous colitis, toxic megacolon, and even death (2–4). The health care burden associated with CDI is estimated to be between $433 million and $4.8 billion annually (5–7). The Food and Drug Administration (FDA) has approved two antibiotics for the treatment of CDI: vancomycin and fidaxomicin (8, 9). Vancomycin, a cell wall synthesis inhibitor, is the treatment of choice in severe and recurrent cases of CDI despite an associated recurrence rate of 20% (9, 10). The Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America (IDSA-SHEA) expert panel defines recurrence as both relapse of the original infection and reinfection with a new strain (9).

Due to an increase in the incidence of CDI and the problem of relapse, new treatment options are urgently needed. With pharmaceutical interest in developing new antibiotics waning to only about 1% (6 out of 506) of new drugs in development (9), another option is to examine whether drugs indicated for other infections could be effective treatments for CDI. One such candidate antibiotic is tigecycline. A derivative of tetracycline and member of the glycylcycline class of antibiotics, tigecycline was FDA approved in 2005 to treat complicated intra-abdominal, skin, and skin structure infections and in 2009 to treat community-acquired pneumonia. Tigecycline reversibly binds the 30S ribosomal subunit at the A site, thereby inhibiting protein synthesis (11). Tigecycline may be an alternate agent to treat patients with CDI, in part due to its low MIC against *C. difficile in vitro* (12). In the clinical setting, tigecycline has been successfully used to treat a small number of patients with severe CDI, with a low incidence of relapse (13–15). However, some clinical reports have found the opposite result (16, 17). With only a few clinical reports and one published mouse study assessing the role of tigecycline in treating CDI (18), more studies are needed to assess the utility of this drug in the treatment of CDI.

To understand how tigecycline affects CDI, we used an established mouse model of CDI in cefoperazone-treated animals (19, 20). These animals were colonized with high levels of *C. difficile* and then were treated with tigecycline and continually monitored for signs of disease. As a comparator, another group of mice was treated with vancomycin, one of the antibiotics currently approved for treatment of CDI. We hypothesized that both tigecycline and vancomycin would be able to treat established *C. difficile* infection in mice; however, the potential for disease following treatment was not known. In this study, we demonstrated that tigecycline and vancomycin prevented the development of clinical disease in experimentally infected mice. This protection occurred

Received 12 September 2014 **Returned for modification** 18 October 2014
Accepted 19 December 2014

Accepted manuscript posted online 29 December 2014

Citation Theriot CM, Schumacher CA, Bassis CM, Seekatz AM, Young VB. 2015. Effects of tigecycline and vancomycin administration on established *Clostridium difficile* infection. Antimicrob Agents Chemother 59:1596–1604. doi:10.1128/AAC.04296-14.

Address correspondence to Casey M. Theriot, caseythe@med.umich.edu.

* Present address: Cassie A. Schumacher, Swift Biosciences, Ann Arbor, Michigan, USA.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04296-14.

doi:10.1128/AAC.04296-14

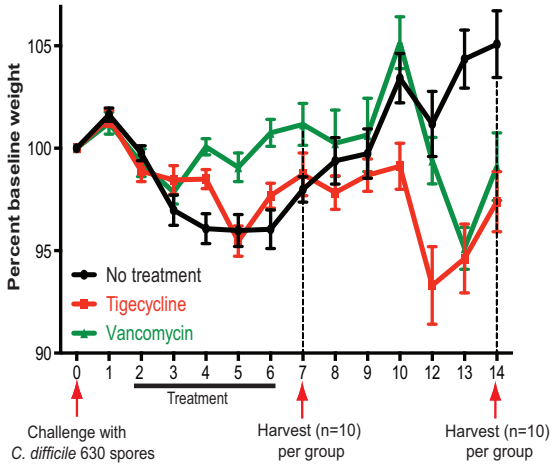

FIG 1 Murine weight loss throughout treatment and development of CDI with tigecycline and vancomycin. Solid lines represent the mean percentage of the baseline weight for animals in each group. After cefoperazone treatment, mice were challenged with *C. difficile* strain 630 spores on day 0 and treated with either tigecycline (red, $n = 10$), vancomycin (green, $n = 10$), or no antibiotics (black, $n = 10$) from day 2 to day 7. Mice from each treatment group ($n = 10$ per group) were euthanized and necropsy was done on days 7 and day 14 postchallenge (dashed lines) to assess if treatment was successful and if disease had occurred. After tigecycline and vancomycin treatment stopped, mice lost a significant amount of body weight between days 10 and 13 compared to untreated controls.

only while the animals were on the antibiotic, as signs of disease developed once the antibiotic course was completed.

MATERIALS AND METHODS

Ethics statement. The University Committee on the Care and Use of Animals (UCUCA) at the University of Michigan approved this study. The University of Michigan laboratory animal care policies follow the Public Health Service policy on humane care and use of laboratory animals. Animals were assessed twice daily for physical condition and behavior, and those assessed as moribund were humanely euthanized by CO_2 asphyxiation. Trained animal technicians performed animal husbandry in an AAALAC-accredited facility.

Animals and housing. Five- to 8-week-old C57BL/6 wild-type (WT) mice (male or female) were obtained from a breeding colony at the University of Michigan that was originally established using animals purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed with autoclaved food, bedding, and water. Cage changes were performed in a laminar flow hood. Mice had a 12-hour cycle of light and darkness.

***C. difficile* strains and antibiotics.** *C. difficile* strain 630 (ATCC BAA-1382) was used in this study. Tigecycline (Pfizer) and vancomycin (Sigma) were dissolved in a 0.9% sodium chloride solution (Hospira). Antibiotic solutions were filter sterilized (0.22 μm; Fisher Scientific) before being added to bacterial cultures.

***C. difficile* spore preparation.** *C. difficile* spores were prepared as previously described (19). Briefly, *C. difficile* strain 630 was grown anaerobically overnight at 37°C from a single colony in a 2-ml culture of Columbia broth. The next day, the inoculum was added to 40 ml of Clospore medium (21). The culture was incubated at 37°C for 5 to 7 days under anaerobic conditions. Spores were harvested by centrifugation and

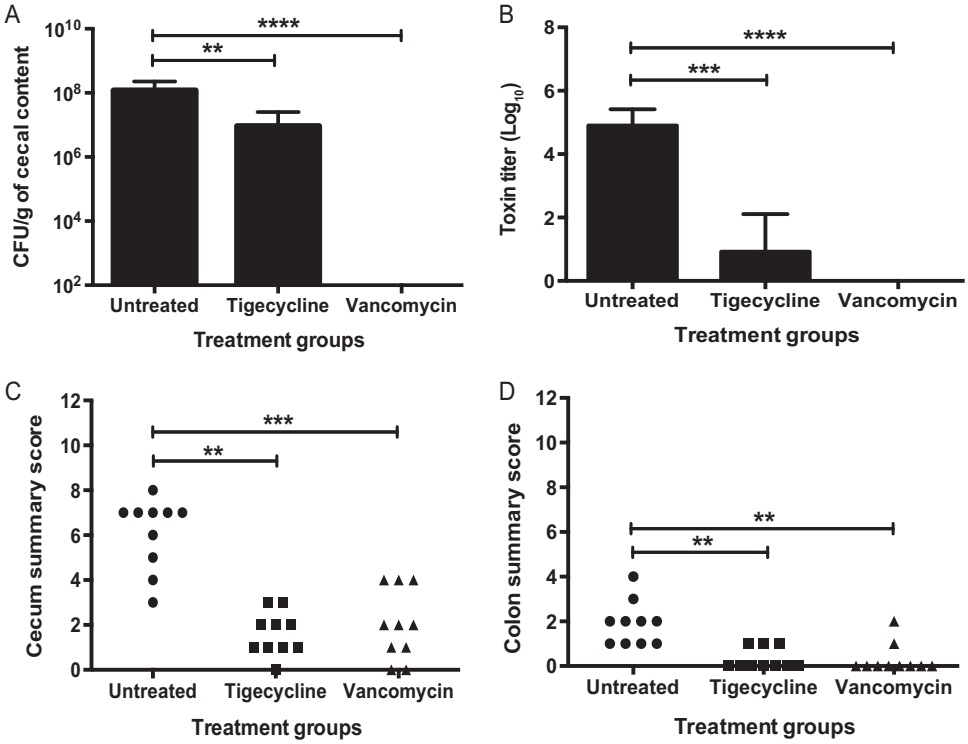

FIG 2 Tigecycline- and vancomycin-treated mice show decreased signs of disease immediately following treatment (day 7 postchallenge). (A) *C. difficile* colonization levels for each of the three treatment groups in CFU per gram of cecal content at the time of necropsy. (B) Vero cell cytotoxicity assay from cecal content of each mouse in $\log_{10}$ reciprocal dilution toxin per gram of cecal content at the time of necropsy. (C and D) Histopathological summary scores of edema, inflammation, and epithelial damage in the murine cecum (C) and colon (D) after each treatment. Error bars depict standard deviation. Significance was determined by the nonparametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (A, C, and D) or followed by Tukey's posttest (B) (**, $P < 0.01$; *** $P < 0.001$; ****, $P < 0.0001$).

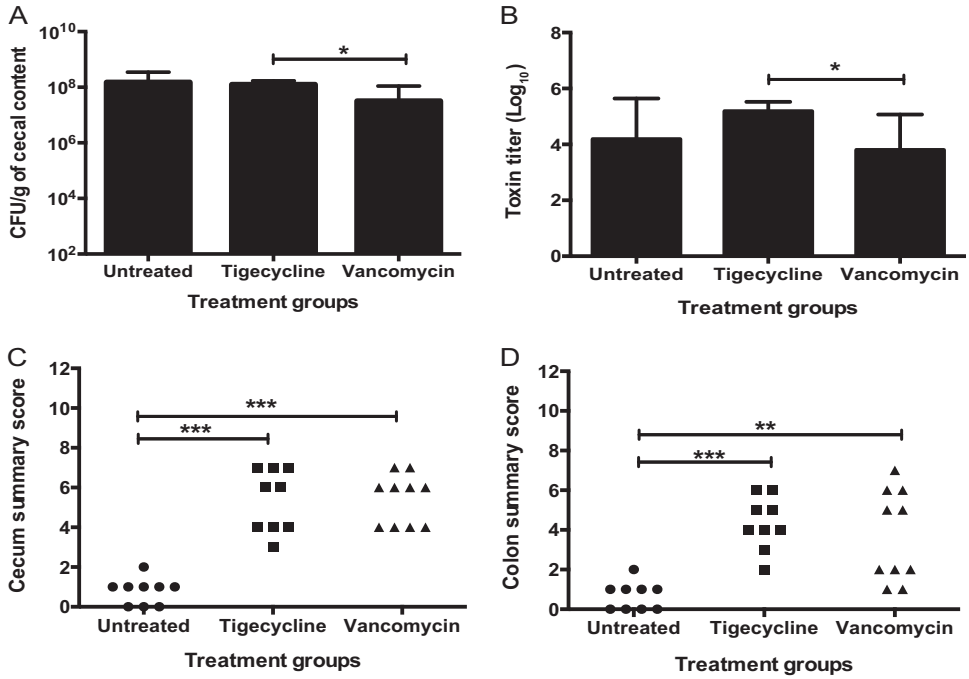

FIG 3 Tigecycline- and vancomycin-treated mice show increased signs of disease 7 days following treatment (day 14 postchallenge). (A) *C. difficile* colonization levels for each of the three treatment groups in CFU per gram of cecal content at the time of necropsy. (B) Vero cell cytotoxicity assay from the cecal content of each mouse in $\log_{10}$ reciprocal dilution toxin per gram of cecal content at the time of necropsy. (C and D) Histopathological summary scores of edema, inflammation, and epithelial damage in the murine cecum (C) and colon (D) 7 days after each treatment. Significance was determined by the nonparametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (A, C, and D) or followed by Tukey's posttest (B) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

washed with cold water at least three times. Spore stocks were stored at 4°C in sterile water. *C. difficile* spores were heat treated for 20 min at 65°C to ensure that any remaining vegetative bacilli were killed prior to gavaging animals. Viable spores were enumerated by plating for CFU/ml on taurocholate, cefoxitin, cycloserine, fructose, and agar (TCCFA).

Antibiotic administration and challenge with *C. difficile* spores. Five- to 8-week-old C57BL/6 mice were treated with a 5-day course of cefoperazone (0.5 mg/ml) in their drinking water followed by a 2-day recovery, at which time mice were orally challenged with approximately 500 *C. difficile* strain 630 spores. After *C. difficile* colonization was established, at 2 days postchallenge, one group of mice ($n = 10$ per group) remained untreated, while another was started on a 5-day course of tigecycline (6.25 mg/kg administered subcutaneously every 12 h) and a third was started on a 5-day course of vancomycin (0.4 mg/ml in drinking water). Vancomycin concentrations were based on previous *C. difficile* studies (22, 23). Mice were evaluated for clinical signs of *C. difficile* infection, including loss of weight, hunched posture, and inappetence. A subset of mice ($n = 10$ per group) was euthanized immediately after the treatment period, while the rest were euthanized 7 days after the end of treatment. At the time of necropsy, the number of *C. difficile* organisms in the cecal contents of infected animals was enumerated by plating on TCCFA selective agar and incubating the plates in an anaerobic environment. Cecal and colonic content and tissue were collected at the time of necropsy and processed for cytotoxicity assays or prepared for histological analysis.

Detection of *C. difficile* toxins via Vero cell cytotoxicity assay. Vero cell cytotoxicity assays were performed as described previously (19). Briefly, Vero cells were grown to confluence in Dulbecco modified Eagle medium (DMEM) (Gibco Laboratories, catalog no. 11965) supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, catalog no. 16140) and 1% penicillin-streptomycin (Gibco Laboratories, catalog no. 15140) at 37°C in 5% CO_2. Cells were then trypsinized (0.25%) (Gibco Laboratories, catalog no. 25200), collected in 4 volumes DMEM, and harvested by centrifugation at 1,000 rpm. Cells were then seeded in a 96-well flat-bottom microtiter plate (Corning, catalog no. 3596) at a density of 1×10^5 cells/well.

To detect toxin in the intestines of infected animals, luminal contents from mouse ceca were weighed and subsequently diluted 1:10 in sterile phosphate-buffered saline (PBS) (Invitrogen). Samples were then vortexed and spun at 13,000 rpm for 5 min. The supernatant was then passed through a 0.22-μm filter. Each filtered sample was then used to make serial 1:10 dilutions in sterile PBS in a 96-well plate. To assess toxin production by *C. difficile* grown *in vitro* culture supernatant, serial 1:10 dilutions were prepared in sterile PBS (Invitrogen). Ten microliters of the diluted samples was added to the Vero cells in a 96-well plate no earlier than 4 hours postplating. Each test well had a corresponding control to which a neutralizing antitoxin antiserum (TechLab, catalog no. T5000) was added in addition to the sample. The plates containing Vero cells and toxin samples were incubated at 37°C in 5% CO_2 overnight. Results were determined the following morning by viewing under a magnification of ×200 for Vero cell rounding. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in at least 80% of Vero cells per gram of cecal sample, provided that this activity was blocked by the addition of antitoxin antiserum in the corresponding control well.

Histological scoring. The histological scoring of the murine cecum and colon was performed as described previously (19). Briefly, coded, randomized slides were scored by a board-certified veterinary pathologist in a blinded manner using the following scoring criteria. Edema scores were as follows: 0, no edema; 1, mild edema with minimal (<2×) multifocal submucosal expansion; 2, moderate edema with moderate (2× to 3×) multifocal submucosal expansion; 3, severe edema with severe (>3×) multifocal submucosal expansion; and 4, same as score 3 with diffuse submucosal expansion. Cellular infiltration scores were as follows: 0, no inflammation; 1, minimal multifocal neutrophilic inflammation; 2, moderate multifocal neutrophilic inflammation (greater submucosal involvement); 3, severe multifocal to coalescing neutrophilic inflammation (greater submucosal with or without mural involvement); and 4, same as

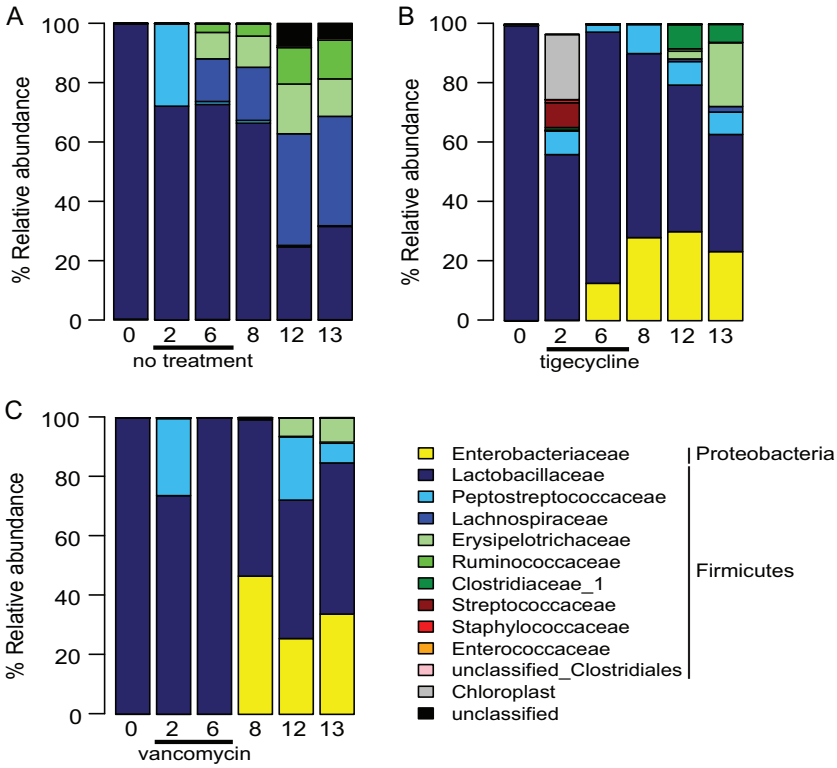

FIG 4 Changes to the murine gut microbiota throughout *C. difficile* infection, treatment, and disease. Bar graphs depict the mean percent abundances of top bacterial families (≥1% relative abundance) throughout *C. difficile* infection alone (untreated) (A) or with tigecycline treatment (B) and vancomycin treatment (C).

score 3 with abscesses or extensive mural involvement. Epithelial damage scores were as follows: 0, no epithelial changes; 1, minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, or villus tip attenuation/necrosis); 3, severe multifocal epithelial damage (same as above) with or without pseudomembrane (intraluminal neutrophils or sloughed epithelium in a fibrinous matrix); 4, same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium). Scores were assigned in all three categories from both cecal and colonic sections. A summary score was then generated for each mouse by adding the scores for each tissue across all 3 categories (edema, inflammation, and epithelial damage) (24, 25).

Microbiome analysis. DNA extraction and sequencing of 16S rRNA-encoding gene amplicons were conducted as previously described (26). Briefly, DNA was isolated from mouse fecal pellets using the MoBio PowerSoil DNA isolation kit optimized for the epMotion (MoBio Laboratories, Inc.) according to the manufacturer's instructions and adapted for the BioMek FXp lab automation workstation (Beckman Coulter). The V3-V5 region of the bacterial 16S rRNA gene was amplified using barcoded sets of primers A 926R and B 357F, which include adapter sequences required for emulsion PCR during 454 sequencing, as described by the Human Microbiome Project (HMP) Consortium's PCR for 454 sequencing. For each 20-μl PCR mixture, 2 μl of AccuPrime PCR buffer II (Life Technologies), 0.15 μl of AccuPrime *Taq* high-fidelity DNA polymerase (Life Technologies), 0.2 μM (each) primers A and B, and 1 μl of DNA sample were added. The PCR cycle was run as previously described (26). The PCR products were purified using AMPure XP (Agencourt) according to the manufacturer's instructions, except 0.6× the amplicon volume of beads was used. Purified PCR products were quantified using the Quant-IT PicoGreen double-stranded DNA (dsDNA) kit (Invitrogen) according to the manufacturer's directions. The barcoded PCR products were pooled and quantified with the Roche 454 GS Titanium (KAPA) sequencing kit (Roche), and 454 sequencing was done using the GS FLX Titanium platform (Roche) according to the manufacturer's instructions.

Sequences were processed and analyzed using Mothur v. 1.29.1 according to the standard operating procedures (SOP) for 454 analyses as of September 2013 (27). Sequences were aligned to the Silva rRNA gene database (28) and were classified with the Mothur-adapted RDP training set v9 (29) using the Wang method and an 80% bootstrap minimum to the family taxonomic level. All samples with <500 sequences were removed. A cutoff of 0.03 (97%) was used to define operational taxonomic units (OTUs) and to calculate the inverse Simpson index as a measure for diversity. Standard packages in R were used to create average bar graphs per mouse treatment group and changes in the inverse Simpson index over time per treatment group.

MIC determination. To determine the MICs of tigecycline for *C. difficile*, spores were plated overnight onto brain heart infusion (BHI) agar supplemented with 1% cysteine and taurocholate and allowed to incubate anaerobically overnight at 37°C. The next evening, one colony from the plate was used to inoculate 5 ml of BHI–1% cysteine broth. The next morning, the culture was back-diluted 1:10 in fresh BHI–1% cysteine broth. After allowing the culture to grow for 4 hours, this culture was used to inoculate a fresh culture of BHI broth to a starting optical density at 600 nm (OD_{600}) of 0.02. Fivefold changes in antibiotic concentrations were used to find the MIC, determined by checking the OD_{600} after 24 hours; inhibition of growth was defined as no change in OD_{600}. To more precisely define the MIC, a range around the previously determined MIC was chosen and the same experiment was repeated, this time using 2-fold dilutions of tigecycline concentrations.

To determine the MICs for vancomycin, the growth conditions were the same as previously described. This time, however, the OD_{600} was measured after 8 hours of growth instead of at 24 hours. This change was made to capture the cells as they grew in log phase instead of trying to

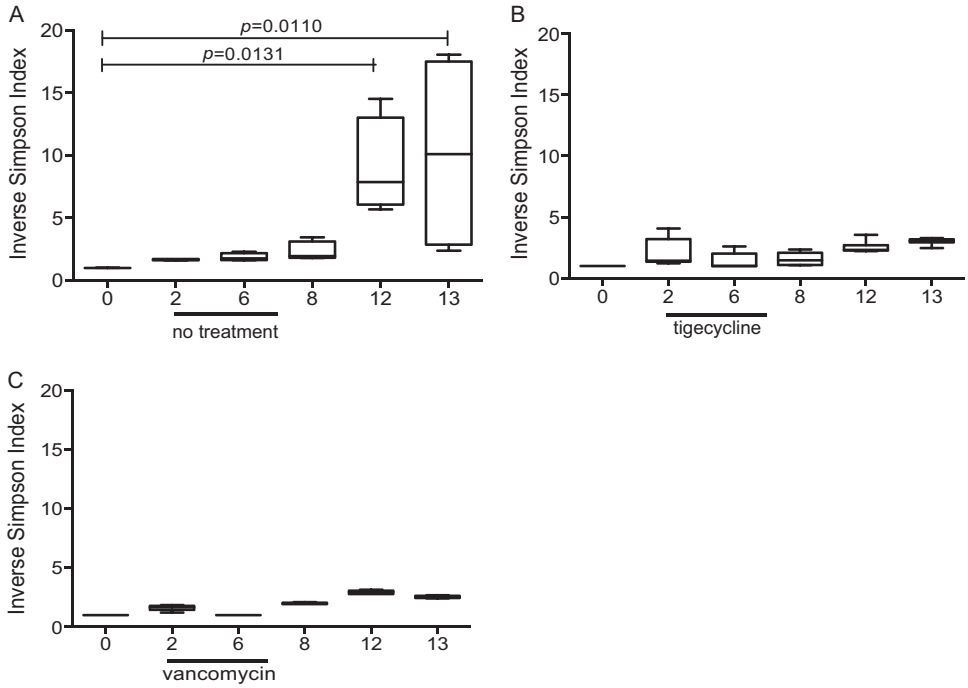

FIG 5 Bacterial diversity increases over time in *C. difficile*-challenged non-antibiotic-treated mice compared to tigecycline- and vancomycin-treated mice. The inverse Simpson index of the gut microbiota throughout *C. difficile* infection alone (untreated) or with tigecycline treatment (B) or vancomycin treatment (C) is shown. Significance was determined by the nonparametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest. Asterisks indicate a significant difference in bacterial diversity between mice at different time points throughout infection (*, $P < 0.05$). The box and whiskers represent the smallest and largest values with the median in the center of each box.

capture them in stationary-phase growth. All steps were carried out in an anaerobic chamber (Coy Laboratory Products).

***C. difficile* growth curve studies.** All steps were carried out in an anaerobic chamber (Coy Laboratory Products). Spores from *C. difficile* strain 630 were plated on BHI agar plates supplemented with 1% cysteine and taurocholate and incubated at 37°C overnight. The next afternoon, one colony was used to inoculate 5 ml BHI–1% cysteine broth. This broth culture was incubated at 37°C overnight. The next morning, 4 ml of the overnight culture was back-diluted in 36 ml fresh BHI–1% cysteine broth. To confirm that the experimental culture was started from an actively growing culture, an initial OD_{600} measurement was taken and then the culture was allowed to grow for 4 hours at 37°C. OD_{600} measurements were obtained from 500-μl samples placed in plastic cuvettes (Denville) and read at 600 nm in a spectrophotometer (Biochrom). After the 4-hour incubation, another OD_{600} measurement was taken. Cells from this 40-ml culture were then added to BHI broth to make 10 ml cultures with an initial OD_{600} of 0.01 to 0.02; this represents time zero. The OD_{600} was monitored for 48 hours. Antibiotics were added after 4 hours of growth.

To assess toxin activity, a 500-μl sample from each culture was passed through a 0.22-μm polyvinylidene difluoride (PVDF) filter (Fisher) and stored at 4°C until the toxin assay could be performed (no more than 48 h later). It is important to note that storing the samples at −20°C has a damaging effect on toxin activity. Samples used for the cytotoxicity assay were collected at 0, 6, 12, 24, and 48 h throughout the growth curve.

Statistical analysis. Kruskal-Wallis one-way analysis of variance (ANOVA) for nonparametric data followed by Dunn's multiple-comparison test was performed using Prism version 6.00c for Mac OS X (GraphPad Software, La Jolla, CA, USA). For comparison of differences between growth of cultures with and without antibiotics, Kruskal-Wallis one-way ANOVA for nonparametric data followed by Dunn's multiple-comparison test was used. For comparison of differences between toxin activity of cultures with and without antibiotics, one-way ANOVA for parametric data followed by Tukey's multiple-comparison test was used. Statistical significance was set at a P value of <0.05 for all analyses.

RESULTS

Treatment of established *C. difficile* with tigecycline and vancomycin. Mice received the antibiotic cefoperazone to make them susceptible to CDI and then were challenged with spores of *C. difficile* strain 630 (Fig. 1) (19). High levels of *C. difficile* were confirmed by bacterial enumeration from fecal contents at 1 day postchallenge (between 10^7 and 10^8 CFU/gram of fecal content). Colonized mice were treated with either a 5-day course of tigecycline (6.25 mg/kg of every 12 h via subcutaneous injection) or vancomycin (0.4 mg/ml in drinking water). An additional control group of infected mice did not receive either antibiotic (Fig. 1). At the end of the treatment period on day 7, a subset of mice from each treatment group ($n = 10$ per group) was sacrificed to evaluate the presence of histopathological disease. The remainder of mice from each treatment group ($n = 10$ per group) were sacrificed 7 days after stopping antibiotics to evaluate signs of disease from CDI (Fig. 1).

Immediately after completing the treatment course on day 7, untreated mice were colonized with high levels of *C. difficile* as measured by culture of cecal contents (Fig. 2A). Mice treated with vancomycin had no detectable levels of *C. difficile* in their ceca upon completion of treatment. Unlike the vancomycin-treated animals, mice treated with tigecycline still had *C. difficile* detectable by culture following treatment, but the organism load was significantly lower than that seen in untreated animals.

After treatment, significantly less cytotoxic activity was seen in

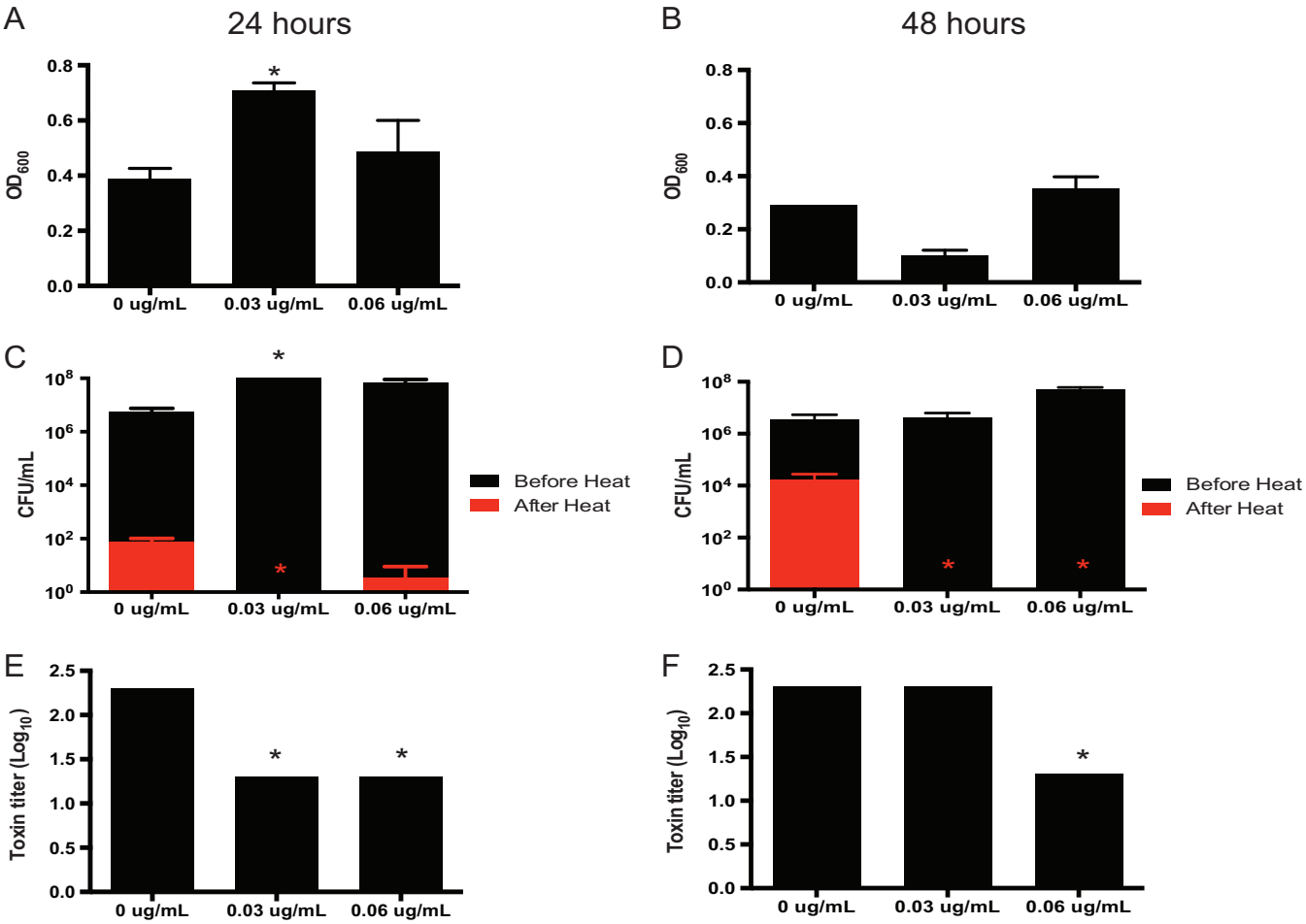

FIG 6 Subinhibitory concentrations of tigecycline inhibit *C. difficile* spore formation and toxin activity. (A and B) Growth of *C. difficile* strain 630 by OD_{600} at 24 h (C) and 48 h (B) in BHI growth medium supplemented with 0, 0.03, or 0.06 μg/ml of tigecycline at 4 h. (C and D) Quantitation of *C. difficile* spores (after heat, in red) and both spores and vegetative cells (before heat, in black) in CFU per ml in cultures at 24 h (C) and 48 h (D). (E and F) Vero cell cytotoxicity of *C. difficile* supernatants at 24 h (E) and (F) 48 h. Significance was determined by the nonparametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (*, $P < 0.05$). Each bar represents the average measurement for three replicate experiments; error bars represent standard deviations.

the ceca of both the tigecycline- and vancomycin-treated animals than in the untreated controls (Fig. 2B). To understand how treatment with each antibiotic impacted the development of CDI, histopathological analyses of the cecum and colon were performed. The histopathological summary score (a sum of edema, inflammation, and epithelial damage) was significantly higher in untreated animals than in the tigecycline- and vancomycin-treated animals in both the cecum and the colon as assessed immediately following treatment (Fig. 2C and D).

We next wanted to determine if *C. difficile*-associated disease would develop after completion of the antibiotic treatments. Significant weight loss developed in mice after completion of treatment with tigecycline and vancomycin, with maximal weight loss occurring 5 to 6 days after the end of treatment (Fig. 1). At the termination of the experiment, 7 days after completing vancomycin or tigecycline treatment, high levels of *C. difficile* were isolated from the cecal contents of all three groups, with no statistically significant difference from levels in the untreated controls (Fig. 3A).

The levels of *C. difficile* cytotoxin activity in the ceca of both the tigecycline- and vancomycin-treated animals were higher at day 14 than at day 7 postchallenge (Fig. 2B and 3B). The histopathological summary scores were significantly higher in the ceca and the colons of tigecycline- and vancomycin-treated animals than in the untreated controls (Fig. 3C and D). Seven days after stopping antibiotics (day 14), both tigecycline- and vancomycin-treated mice developed *C. difficile*-associated disease.

Alterations in the gut microbiota throughout treatment and disease development. In addition to assessing *C. difficile* colonization, toxin production, and disease dynamics following these treatments in this study, we also defined the gut microbiota of mice during antibiotic treatment and *C. difficile*-associated disease development. After cefoperazone treatment but prior to *C. difficile* challenge, all mice were dominated by bacteria from the *Lactobacillaceae* family (Fig. 4). *C. difficile* from the *Peptostreptococceae* family was detected in all mice at day 2 postchallenge, the first day of treatment, and decreased only during vancomycin treatment. We observed bacterial community differences following different treatments. In untreated mice, several members from the *Firmicutes* phylum (*Ruminococcaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae*) were detectable by day 6 postchallenge (Fig. 4A). Conversely, these microbial families were not detectable in anti-

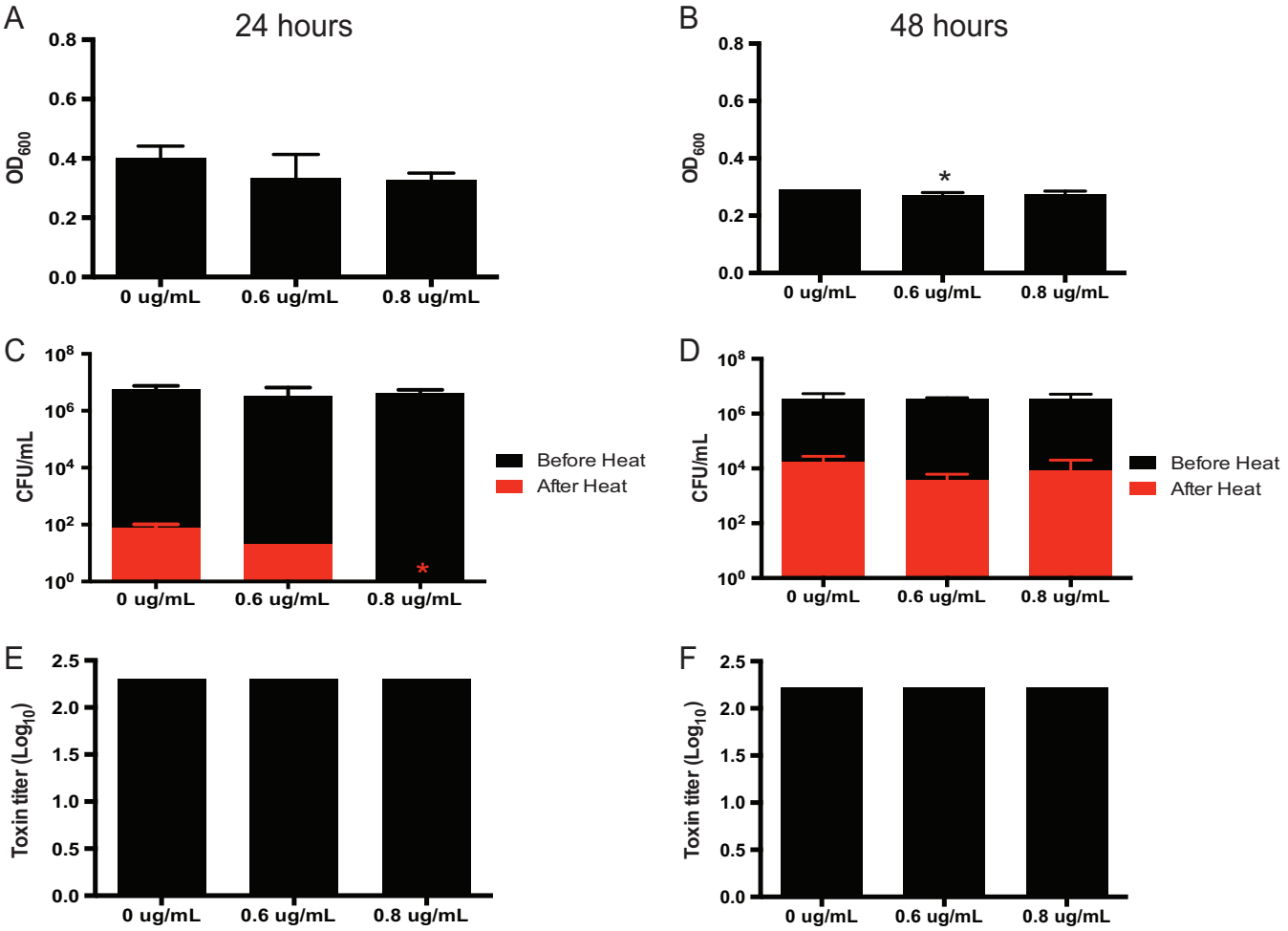

FIG 7 Subinhibitory concentrations of vancomycin do not inhibit *C. difficile* spore formation and toxin activity. (A and B) Growth of *C. difficile* strain 630 by OD_{600} at 24 h (A) and 48 h (B) in BHI growth medium supplemented with 0, 0.6, or 0.8 μg/ml of vancomycin at 4 h. (C and D) Quantitation of *C. difficile* spores (after heat, in red) and both spores and vegetative cells (before heat, in black) in CFU per ml in cultures at 24 h (C) and 48 h (D). (E and F) Vero cell cytotoxicity of *C. difficile* supernatants at 24 h (E) and 48 h (F). Significance was determined by the nonparametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (*, $P < 0.05$). Each bar represents the average measurement for three replicate experiments; error bars represent standard deviations.

biotic-treated mice until day 12 postchallenge. Instead, both antibiotic-treated mouse groups exhibited an increased abundance of the *Enterobacteriaceae* family immediately following cessation of the antibiotic treatment (Fig. 4B and C). A similar trend was observed in the bacterial diversity over time. Untreated mice exhibited an increase in diversity by day 12-postchallenge as measured by the inverse Simpson index, whereas antibiotic-treated mice did not recover diversity after antibiotic treatment (Fig. 5). These data suggest that both tigecycline and vancomycin further altered the gut microbiota, delaying the recovery of the bacterial community following their use.

Subinhibitory concentrations of tigecycline decrease *C. difficile* spore formation and toxin activity but not growth *in vitro*. To investigate the discordance between the high levels of *C. difficile* colonization and low levels of cytotoxicity and histopathology in tigecycline-treated mice at day 7, we used an *in vitro* approach. We determined the MIC of tigecycline in broth culture with *C. difficile* strain 630 to be 0.06 μg/ml. *In vitro* growth curves were done, and after 4 h of *C. difficile* growth, two concentrations of tigecycline (0.03 and 0.06 μg/ml) were supplemented into the growth medium. These concentrations were selected based on growth curve studies that demonstrated that they do not decrease the growth of *C. difficile* but do decrease the toxin activity (see Fig. S1A and C in the supplemental material). As expected, in this experiment neither tigecycline concentration decreased *C. difficile* growth by 24 or 48 h of growth (Fig. 6A and B). By 24 h, there was a significant decrease in spore formation at the 0.03-μg/ml concentration and in cytotoxicity at both concentrations (Fig. 6C and E). After 48 h of growth, both concentrations of tigecycline significantly decreased spore formation, while cytotoxicity was significantly decreased only at the 0.06 μg/ml concentration (Fig. 6D and F).

We next determined the MIC of vancomycin in broth culture with *C. difficile* strain 630 to be 0.8 μg/ml. *In vitro* growth curves were done, and after 4 h of *C. difficile* growth, two concentrations of vancomycin (0.6 and 0.8 μg/ml) were supplemented into the growth medium. These concentrations were selected based on previous growth studies showing that they do not decrease the growth of *C. difficile* or cytotoxicity (see Fig. S1B and D in the supplemental material). Neither concentration of vancomycin

decreased *C. difficile* growth or cytotoxicity by the 24-h time point (Fig. 7A and E). After 24 h of growth, there was a significant decrease in spore formation at the 0.8-μg/ml vancomycin concentration (Fig. 7C). However, in contrast to the case for tigecycline, neither concentration of vancomycin affected spore formation or cytotoxicity at 48 h (Fig. 7D and F).

DISCUSSION

The recent resurgence of severe *C. difficile* infection has prompted the search for alternative treatments for this devastating nosocomial infection. Part of the search involved testing existing antibiotics that have *in vitro* activity against *C. difficile*. Tigecycline is one such antibiotic that has been used in case studies and shown to have efficacy in the setting of refractory disease. However, no clinical trials of this drug have been attempted. We used a mouse model of CDI to show that treatment with tigecycline can significantly decrease the severity of disease associated with *C. difficile* colonization. Tigecycline treatment of infected mice resulted in decreased intestinal damage that was associated with lower levels of *C. difficile* cytotoxic activity without a substantial change in organism load. These results are consistent with the mechanism of action of the drug, which inhibits protein synthesis. Treatment of *C. difficile*-infected mice with the comparator drug vancomycin decreased both pathogen load and toxin activity. These results are consistent with a previous study by Jump et al. in which tigecycline treatment did not suppress levels of *C. difficile* compared to those with vancomycin treatment in colonized mice. However, it is important to note that disease due to *C. difficile* infection was not monitored in this study (18).

Interestingly, in our model both antibiotics were able to delay the onset of disease in two different manners, one by suppressing toxin and the other by significantly decreasing the *C. difficile* load. We went on to confirm these results using an *in vitro* approach. Subinhibitory concentrations of tigecycline were able to significantly modulate toxin activity and spore formation without affecting *C. difficile* growth *in vitro*. This is in agreement with other *in vitro* studies in which subinhibitory concentrations of tigecycline were able to decrease both toxin production and spore formation by certain epidemic *C. difficile* strains (30, 31). Understanding how antibiotics target the *C. difficile* life cycle both *in vitro* and, more importantly, *in vivo* could lead to more targeted treatment options for patients with CDI.

In this study, we also looked at the gut microbiota of mice throughout treatment and disease development. Untreated mice were able to recover from CDI over time and were associated with increased bacterial diversity, while treated mice had further disruption of the gut microbiota with tigecycline and vancomycin treatment. After antibiotic pressure was lifted, mice were still in a state susceptible to CDI, which could explain why they developed disease. Alterations in the gut microbiota from antibiotics used to treat CDI patients have been associated with recurrent CDI (32). It is important to understand how antibiotics used for treatment of CDI are able to alter the *C. difficile* life cycle but also how they further alter the gut microbiota.

In summary, there is a need to discover new treatments for severe and refractory CDI. Currently, the only FDA-approved antimicrobials for treating CDI are vancomycin and fidaxomicin. Based on the current study, tigecycline could play a role in treating patients with CDI by suppressing toxin activity and spore formation, which would alleviate disease. Even though this has been demonstrated *in vitro* and now in a mouse model of CDI, further animal studies, including the hamster model, are needed to evaluate the effectiveness of tigecycline against CDI.

ACKNOWLEDGMENTS

We thank Kathy Wozniak and the University of Michigan ULAM staff for help with the mouse work. We also thank Sue Foltin and Gabrielle Hatton for help with the DNA extractions and 454 pyrosequencing. We thank the Microbial Systems Laboratory of the Host Microbiome Initiative for sequencing support.

Funding for this project comes from Pfizer (Advancing Science through Pfizer-Investigator Research Exchange [ASPIRE], grant 673305) and NIH grant U19AI090871. C.M.T. is supported by the NIH Metabolomics Common Fund and National Institute of General Medical Science under award K01GM109236. A.M.S. is supported by grant 2UL1TR000433 from the National Center for Advancing Translational Sciences.

REFERENCES

1. **Hall JC, O'Toole E.** 1935. Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am J Dis Child **49:**390–402. http://dx.doi.org/10.1001/archpedi.1935.01970020105010.
2. **McCollum DL, Rodriguez JM.** 2012. Detection, treatment, and prevention of *Clostridium difficile* infection. Clin Gastroenterol Hepatol **10:**581–592. http://dx.doi.org/10.1016/j.cgh.2012.03.008.
3. **Stanley JD, Bartlett JG, Dart BW, IV, Ashcraft JH.** 2013. Clostridium difficile infection. Curr Probl Surg **50:**302–337. http://dx.doi.org/10.1067/j.cpsurg.2013.02.004.
4. **Bartlett JG.** 1984. Antibiotic-associated colitis. Dis Mon **30:**1–54. http://dx.doi.org/10.1016/0011-5029(84)90009-9.
5. **Dubberke ER, Wertheimer AI.** 2009. Review of current literature on the economic burden of *Clostridium difficile* infection. Infect Control Hosp Epidemiol **30:**57–66. http://dx.doi.org/10.1086/592981.
6. **Ghantoji SS, Sail K, Lairson DR, DuPont HL, Garey KW.** 2010. Economic healthcare costs of *Clostridium difficile* infection: a systematic review. J Hosp Infect **74:**309–318. http://dx.doi.org/10.1016/j.jhin.2009.10.016.
7. **Dubberke ER, Olsen MA.** 2012. Burden of *Clostridium difficile* on the healthcare system. Clin Infect Dis **55**(Suppl 2)**:**S88–S92. http://dx.doi.org/10.1093/cid/cis335.
8. **U.S. FDA.** 2011. FDA approves treatment for *Clostridium difficile* infection. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm257024.htm.
9. **Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH.** 2010. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Infect Control Hosp Epidemiol **31:**431–455. http://dx.doi.org/10.1086/651706.
10. **Reynolds PE.** 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis **8:**943–950. http://dx.doi.org/10.1007/BF01967563.
11. **Kasbekar N.** 2006. Tigecycline: a new glycylcycline antimicrobial agent. Am J Health System Pharm **63:**1235–1243. http://dx.doi.org/10.2146/ajhp050487.
12. **Hecht DW, Galang MA, Sambol SP, Osmolski JR, Johnson S, Gerding DN.** 2007. In vitro activities of 15 antimicrobial agents against 110 toxigenic *Clostridium difficile* clinical isolates collected from 1983 to 2004. Antimicrob Agents Chemother **51:**2716–2719. http://dx.doi.org/10.1128/AAC.01623-06.
13. **Herpers BL, Vlaminckx B, Burkhardt O, Blom H, Biemond-Moeniralam HS, Hornef M, Welte T, Kuijper EJ.** 2009. Intravenous tigecycline as adjunctive or alternative therapy for severe refractory *Clostridium difficile* infection. Clin Infect Dis **48:**1732–1735. http://dx.doi.org/10.1086/599224.
14. **Lu C-L, Liu C-Y, Liao C-H, Huang Y-T, Wang H-P, Hsueh P-R.** 2010. Severe and refractory *Clostridium difficile* infection successfully treated with tigecycline and metronidazole. Int J Antimicrob Agents **35:**311–312. http://dx.doi.org/10.1016/j.ijantimicag.2009.11.008.
15. **Hawser SP.** 2010. Activity of tigecycline against recent European clinical isolates of *Clostridium difficile*. Int J Antimicrob Agents **35:**97–98. http://dx.doi.org/10.1016/j.ijantimicag.2009.09.009.

16. **Kopterides P, Papageorgiou C, Antoniadou A, Papadomichelakis E, Tsangaris I, Dimopoulou I, Armaganidis A.** 2010. Failure of tigecycline to treat severe *Clostridium difficile* infection. Anaesthesia Intens Care **38:**755.
17. **Thomas A, Khan F, Uddin N, Wallace MR.** 2014. Tigecycline for severe *Clostridium difficile* infection. Int J Infect Dis **26:**171–172. http://dx.doi.org/10.1016/j.ijid.2014.04.025.
18. **Jump RLP, Li Y, Pultz MJ, Kypriotakis G, Donskey CJ.** 2011. Tigecycline exhibits inhibitory activity against *Clostridiumdifficile* in the colon of mice and does not promote growth or toxin production. Antimicrob Agents Chemother **55:**546–549. http://dx.doi.org/10.1128/AAC.00839-10.
19. **Theriot CM, Koumpouras CC, Carlson PE, Bergin II, Aronoff DM, Young VB.** 2011. Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains. Gut Microbes **2:**326–334. http://dx.doi.org/10.4161/gmic.19142.
20. **Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection. Gut Microbes **2:**145–158. http://dx.doi.org/10.4161/gmic.2.3.16333.
21. **Perez J, Springthorpe VS, Sattar SA.** 2011. Clospore: a liquid medium for producing high titers of semi-purified spores of *Clostridium difficile*. J AOAC Int **94:**618–626.
22. **Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP.** 2008. A mouse model of *Clostridium difficile*-associated disease. Gastroenterology **135:**1984–1992. http://dx.doi.org/10.1053/j.gastro.2008.09.002.
23. **Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, Goulding D, Rad R, Schreiber F, Brandt C, Deakin LJ, Pickard DJ, Duncan SH, Flint HJ, Clark TG, Parkhill J, Dougan G.** 2012. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. PLoS Pathog **8:**e1002995. http://dx.doi.org/10.1371/journal.ppat.1002995.
24. **Holland T.** 2001. A survey of discriminant methods used in toxicological histopathology. Toxicol Pathol **29:**269–273. http://dx.doi.org/10.1080/019262301317052567.
25. **Holland T.** 2005. The comparative power of the discriminant methods used in toxicological pathology. Toxicol Pathol **33:**490–494. http://dx.doi.org/10.1080/01926230590965382.
26. **Bassis CM, Theriot CM, Young VB.** 2014. Alteration of the murine gastrointestinal microbiota by tigecycline leads to increased susceptibility to *Clostridium difficile* infection. Antimicrob Agents Chemother **58:**2767–2774. http://dx.doi.org/10.1128/AAC.02262-13.
27. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75:**7537–7541. http://dx.doi.org/10.1128/AEM.01541-09.
28. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.** 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res **41:**D590–D596. http://dx.doi.org/10.1093/nar/gks1219.
29. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **73:**5261–5267. http://dx.doi.org/10.1128/AEM.00062-07.
30. **Aldape MJ, Heeney DD, Bryant AE, Stevens DL.** 2015. Tigecycline suppresses toxin A and B production and sporulation in *Clostridium difficile*. J Antimicrob Chemother **70:**153–159. http://dx.doi.org/10.1093/jac/dku325.
31. **Garneau JR, Valiquette L, Fortier LC.** 2014. Prevention of *Clostridium difficile* spore formation by sub-inhibitory concentrations of tigecycline and piperacillin/tazobactam. BMC Infect Dis **14:**29. http://dx.doi.org/10.1186/1471-2334-14-29.
32. **Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB.** 2008. Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. J Infect Dis **197:**435–438. http://dx.doi.org/10.1086/525047.